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Review

Targeting Bcl-2 based on the interaction of its BH4 domain with the inositol 1,4,5-trisphosphate receptor

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ABSTRACT

Bcl-2 is the founding member of a large family of apoptosis regulating proteins. Bcl-2 is a prime target for novel therapeutics because it is elevated in many forms of cancer and contributes to cancer progression and therapy resistance based on its ability to inhibit apoptosis. Bcl-2 interacts with proapoptotic members of the Bcl-2 family to inhibit apoptosis and small molecules that disrupt this interaction have already entered the cancer therapy arena. A separate function of Bcl-2 is to inhibit Ca^{2+} signals that promote apoptosis. This function is mediated through interaction of the Bcl-2 BH4 domain with the inositol 1,4,5-trisphosphate receptor (IP3R) Ca^{2+} channel. A novel peptide inhibitor of this interaction enhances proapoptotic Ca^{2+} signals. In preliminary experiments this peptide enhanced ABT-737 induced apoptosis in chronic lymphocytic leukemia cells. These findings draw attention to the BH4 domain as a potential therapeutic target. This review summarizes what is currently known about the BH4 domain of Bcl-2, its interaction with the IP3R and other proteins, and the part it plays in Bcl-2's anti-apoptotic function. In addition, we speculate on how the BH4 domain of Bcl-2 can be targeted therapeutically not only for diseases associated with apoptosis resistance, but also for diseases associated with accelerated cell death.

1. Introduction

Bcl-2 is one of the most important apoptosis regulators. It was first identified through genetic analysis of B cell lymphomas two decades ago and is a major target of novel therapeutic approaches for cancer and other diseases [1–3]. In the most common type of human lymphoma, the region of chromosome 18 encoding Bcl-2 translocates to chromosome 14, downstream of the antibody heavy chain enhancer. Thus, in these *t*(14; 18) positive cells, Bcl-2 expression is driven by the IgH enhancer [4,5]. Bcl-2 is elevated in many cancers, including breast cancer, colon cancer, prostate cancer, small cell lung cancer, chronic lymphocytic leukemia and low-grade lymphomas. The *t*(14; 18) translocation is the mechanism only in lymphomas and a variety mechanisms contribute to Bcl-2 dysregulation and over-expression in other types of cancer [2,6]. The Bcl-2 protein exerts its oncogenic effects at least in part by inhibiting apoptosis. Impaired apoptosis is a crucial step in tumorigenesis, neoplastic progression, metastasis and chemotherapy resistance.

The Bcl-2 family is generally divided into two categories: pro-survival Bcl-2 proteins including Bcl-2, Bcl-xL, A1 and Mcl1; and proapoptotic Bcl-2 proteins including Bax/Bak, Bim, Bad, Bik and Puma [4,5]. The Bcl-2 family members in these two categories can interact with each other. For example, Bcl-2/Bcl-xL can interact with Bim, thereby inhibiting Bim activation induced apoptosis. The balance between pro-survival and proapoptotic Bcl-2 family proteins is a major factor in determining whether or not cells undergo apoptosis in response to cell stress. Disorders of the apoptotic machinery can result in either undesirable cell accumulation as in cancer, or a loss of cells as seen in neurodegenerative, autoimmune and cardiovascular diseases [7]. Modulation of apoptosis has become a novel therapeutic concept. As one of the major apoptosis regulators, Bcl-2 has attracted considerable interest on the part of those involved in developing innovative therapies for cancer [3,8]. These efforts have mainly targeted the inhibitory interaction of Bcl-2 with proapoptotic members of the Bcl-2 protein family, with the goal of disrupting this interaction and thereby abrogating the antiapoptotic action of Bcl-2.

In addition to inhibiting apoptosis by interacting with proapoptotic Bcl-2 relatives, Bcl-2 also interacts with other apoptosis regulators that are not members of the Bcl-2 family. These two different types of interactions take place on different structural domains of the Bcl-2 protein. Interactions with non-Bcl-2 family members involve the BH4 domain, which is located near the N-terminus and is absent from proapoptotic Bcl-2 family members. Here we review recent evidence

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that the BH4 domain of Bcl-2 mediates an interaction between Bcl-2 and a number of factors involved in regulating cell growth and survival, including the inositol 1, 4, 5-trisphosphate receptor (IP3R) intracellular Ca^{2+} channel. Ca^{2+} signals play an important role in mediating apoptosis [9–11]. By interacting with the IP3R Bcl-2 represses Ca^{2+} signals that mediate apoptosis [12,13]. We propose that interactions involving the BH4 domain of Bcl-2 may also prove to be a worthwhile target for therapeutic development.

2. Structure of Bcl-2 proteins

Based on sequence alignment Bcl-2 family members share considerable similarity in regions known as Bcl-2 homology domains (BH domains) [14,15], shown in Fig. 1A. The antiapoptotic family members including Bcl-2, Bcl-xL, and Bcl-w have four BH domains (BH1-BH4), whereas the proapoptotic family members Bax and Bak have only BH1, BH2 and BH3 domains. A third group that includes for example Bim, Bad and Bik, only have a BH3 domain and thus are referred to as BH3-only proteins.

The first X-ray and NMR structure of a Bcl-2 family protein to be determined was that of Bcl-xL [16]. The three dimensional structure of Bcl-2 was subsequently determined by NMR spectroscopy of a Bcl-2/Bcl-xL chimeric protein which contains a truncated version of the unstructured loop between the BH4 and BH3 domains [17]. The N-terminus of Bcl-xL contains an amphipathic α-helical BH4 domain and is located on the protein surface. This domain forms extensive hydrophobic interactions with the α2, α5 and α6 helices located in the region of the BH1, 2 and 3 domains [16]. The C-terminus of Bcl-2 contains a hydrophobic transmembrane region that inserts into intracellular membranes, including mitochondria and endoplasmic reticulum (ER), with the bulk of the Bcl-2 protein oriented on the cytoplasmic face of these organelles. The BH1 and BH2 domains of Bcl-2 are critical for heterodimerization with Bax and important for Bcl-2's pro-survival function [14]. The BH1, BH2 and BH3 domains form a hydrophobic cleft on both the antiapoptotic Bcl-2/Bcl-xL proteins and proapoptotic proteins, Bax and Bak. This binding pocket on the antiapoptotic proteins Bcl-2 and Bcl-xL can be occupied by the α-helix of interacting BH3-only proteins, such as Bim and Bad. Through this mechanism Bcl-2/Bcl-xL sequester the BH3-only proteins, thereby preventing them from activating full-length proapoptotic proteins including Bax and Bak, although an alternative theory is that the prosurvival functions of Bcl-2/Bcl-xL are neutralized by the BH3-only proteins.

3. The antiapoptotic function of Bcl-2

One of the characteristics of Bcl-2 family proteins is their ability to form heterodimers or homodimers, which contributes to the neutralizing competition between antiapoptotic and proapoptotic members. The detailed mechanisms accounting for Bcl-2's antiapoptotic action are not fully understood and consequently there are many hypotheses. As already noted above, considerable attention has focused on the direct interactions between various Bcl-2 family members. It is generally held that through this process, the antiapoptotic proteins Bcl-2 and Bcl-xL prevent Bax and Bak from forming pores in the outer mitochondrial membrane that release factors such as cytochrome *c* [18,19]. These factors, in turn, activate caspases, proteases that function to dismantle the cell during apoptosis. Also, as mentioned above, Bcl-2 and Bcl-xL appear to sequester proapoptotic BH3-only proteins such as Bim and Bad, preventing them from conveying a death signal. Thus, the major current working models of apoptosis control are based on Bcl-2 family members' mutual regulation [20–22]. Many Bcl-2 inhibitors intended for therapeutic application target these interactions, as discussed below.

Although the role of Bcl-2 family members in regulating mitochondrial membrane permeability have been generally emphasized in the apoptosis literature, the roles of Bcl-2 family members on the endoplasmic reticulum (ER) have received increasing attention in

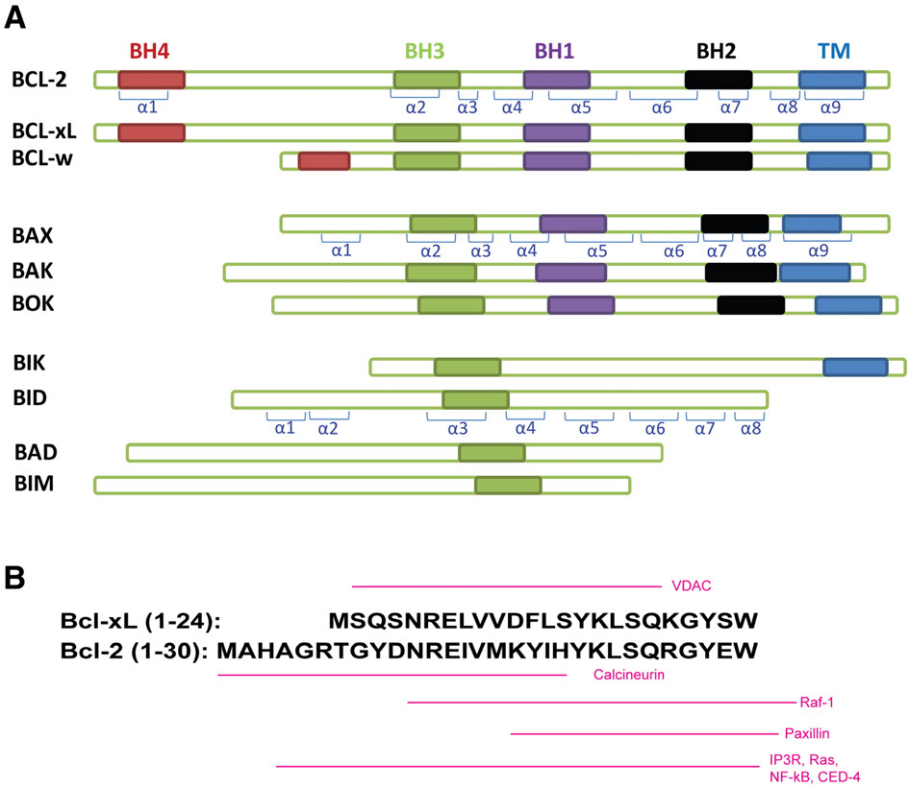

Fig. 1. (A) BH domains of Bcl-2 family members. Bcl-2 family members share sequence similarity in Bcl-2 homology (BH) domains shown by different colored regions. Prosurvival Bcl-2 family members, Bcl-2, Bcl-xL and Bcl-w have all four BH domains, whereas proapoptotic Bax subfamily members do not have a BH4 domain. BH3-only subfamily members lack all but a BH3 domain. Known α-helical regions are indicated. TM, transmembrane domain. (B) The binding regions of different proteins on the N-terminus of Bcl-2 (BH4 domain).

recent years, mainly focused on the role of Bcl-2 family members in regulating Ca^{2+} release from ER. This Ca^{2+} release elevates cytoplasmic Ca^{2+}, producing Ca^{2+} signals that govern many cellular processes, including proliferation, development, cell cycle and apoptosis. The Ca^{2+} ion is a versatile second messenger that regulates life and death signals [23,24]. It induces apoptosis either by direct effects on mitochondria or indirectly by activating or inducing other proapoptotic proteins, including Bim, Bad, proteases and endonucleases [10,11,25]. Bcl-2 represses apoptosis by inhibiting Ca^{2+} release from ER. Two proposed mechanisms for how Bcl-2 regulates ER Ca^{2+}, brought out in the past decade, are the inhibition of IP3R channel opening and the reduction of ER luminal Ca^{2+} concentration (reviewed in [9,26]).

Recent studies indicate that Bcl-2 and Bcl-xL interact with the IP3R Ca^{2+} channel on ER membrane and regulate its activity, thereby inhibiting proapoptotic sustained Ca^{2+} elevation without interfering with prosurvival Ca^{2+} oscillations [12,13,27–31]. Much of this work has been performed in T cells, in which T cell receptor activation (e.g., by antibody to the CD3 component of the T cell receptor complex) can trigger apoptosis, mediated in part through IP3-mediated Ca^{2+} elevation. Intriguingly, a Bcl-2 mutant (G145A) which does not form heterodimers with proapoptotic members of the Bcl-2 family, such as Bax, still protects T cells from anti-CD3-induced apoptosis [32], suggesting that Bcl-2 inhibits this form of apoptosis by a mechanism other than that involving interactions between Bcl-2 family members. One candidate mechanism is the regulation of IP3-mediated Ca^{2+} elevation, based on our evidence that Bcl-2 inhibits anti-CD3-induced apoptosis by inhibiting IP3R-mediated Ca^{2+} release from the ER in T cells [12,27]. Thus, Bcl-2 can inhibit apoptosis independent of, or in addition to, its association with Bax or other proapoptotic Bcl-2 family members in those situations where IP3-mediated Ca^{2+} elevation contributes to apoptosis induction.

Although most recent reports emphasize the interaction of Bcl-2 with other family members, or more recently with the IP3R, Bcl-2 has been reported to also interact with a number of other proteins with the potential of regulating apoptosis, including protein phosphatase 1α, calcineurin, NF-κB, c-myc, FKBP38, Raf-1, NALP1, and Nur77/TR3. Thus, a network of protein–protein interactions may contribute to Bcl-2's antiapoptotic function in cells.

4. The BH4 domain of Bcl-2/Bcl-xL is crucial for its antiapoptotic function

The BH4 domain is highly conserved among antiapoptotic proteins and across species. Thus, there is considerable sequence homology between the BH4 domains of Bcl-2 and Bcl-xL and swapping the BH4 domains between these proteins does not diminish their antiapoptotic function [33]. The BH4 region is the only conserved domain among Bcl-2 family members that is present only in those members with antiapoptotic activity, including Bcl-2, Bcl-xL and Bcl-w, but absent from proapoptotic family members (Fig. 1A). This fact alone suggests that the BH4 domain plays a critical role in determining Bcl-2's antiapoptotic potential. This concept is confirmed by evidence that BH4 domain deletion or mutation eliminates the prosurvival activity of Bcl-2 [34–36] without interfering with the ability of Bcl-2 to bind BH3-only proteins with an affinity similar to that of full length Bcl-2 [33]. Therefore, the BH4 domain-mediated antiapoptotic function of Bcl-2 appears to be independent of its ability to dimerize with other proapoptotic Bcl-2 family members. BH4 deleted Bcl-2 (ΔBH4 Bcl-2) lacks protective function in multiple forms of apoptosis, including that induced by IL-3 deprivation, staurosporine, γ-irradiation and dexamethasone [33,34]. Also, ΔBH4 Bcl-2 may possibly function as a dominant negative inhibitor of Bcl-2 [34]. Others have suggested that ΔBH4 Bcl-2 functions like Bax to promote rather than inhibit cell death [37].

5. BH4 domain interacting proteins

The BH4 domain represents a critical region within the Bcl-2 molecule for the prevention of apoptosis through its ability to interact with some of the following apoptotic regulation proteins.

5.1. Calcineurin

Calcineurin is reported to interact with the BH4 domain of Bcl-2 in baby hamster kidney cells, Jurkat cells and SUDHL-4 B-cell lymphoma cells [38,39]. Amino acids 1–20 at the N terminus of Bcl-2 (Fig. 1B), located in the BH4 domain, are sufficient and necessary to interact with calcineurin. Bcl-2 sequesters active calcineurin from NF-AT, thereby inhibiting calcineurin-dependent NF-AT signaling. Interestingly, the proapoptotic protein Bax interferes with the interaction between Bcl-2 and calcineurin. It is still not clear if the Bcl-2-calcineurin interaction disrupts activation induced cell death in T cells or contributes to lymphoproliferation.

Calcineurin is reported to be anchored not only to Bcl-2 but also to the IP3R. The interaction of calcineurin with the IP3R has been reported and is thought to regulate the phosphorylation status of the receptor, resulting in a Ca^{2+}-sensitive regulation of IP3-mediated Ca^{2+} flux [40]. The regulation of IP3R-mediated Ca^{2+} signaling by calcineurin has been most extensively characterized in the immune system [41–43]. Also, in cortical and hippocampal slices of the brain Billingsley and coworkers reported that calcineurin interacts with both Bcl-2 and the IP3R, proposing that calcineurin shuttles between these two proteins [44,45]. Thus, it is suggested that this triple protein complex contributes to cell survival in primary neuronal cells. However, others have questioned the relevance of calcineurin to IP3R function [41]. Thus, at present the role of calcineurin in the function of both Bcl-2 and the IP3R remains uncertain and requires further clarification.

5.2. VDAC

The IP3R is not the only intracellular channel with which antiapoptotic Bcl-2 family members interact. The BH4 domain of Bcl-xL has been reported to interact with the voltage dependent anion channel (VDAC), which is located on mitochondria and responsible for regulating mitochondrial membrane potential [46–48]. The interaction of the BH4 domain with VDAC is required for Bcl-xL to inhibit etoposide-induced cytochrome *c* release and preserve VDAC channel activity and mitochondrial membrane potential [49]. Although the N-terminus of Bcl-xL (amino acids 2–19aa) coimmunoprecipitates with VDAC, ΔBH4 Bcl-xL still interacts with VDAC, suggesting that the BH4 domain is not necessary for Bcl-xL–VDAC interaction [49]. BH4 peptide's antiapoptotic function has been attributed to the inhibition of VDAC channel activity, thereby preventing apoptotic mitochondrial changes [49]. But knocking down VDAC has no effect on mitochondrial permeability transition and cell death, indicating that VDAC is dispensable for mitochondria-dependent cell death [50]. This has led some to question the importance of the VDAC channel in mitochondria-mediated apoptosis, and hence to question whether the interaction between Bcl-xL and VDAC contributes significantly to Bcl-xL's antiapoptotic function.

5.3. Raf-1

Raf-1, a serine/threonine protein kinase in the MAPK/ERK signal transduction pathway, plays important roles in cell cycle, apoptosis, and differentiation [51,52]. It has been reported to interact with the BH4 domain of Bcl-2 (residues 11–33) (Fig. 1B) and in this manner to regulate apoptosis [53,54], although this interaction may not be stable in cellular extracts [55]. One theory is that Bcl-2 targets Raf-1 to mitochondria to block cell death by phosphorylating the BH3-only

proapoptotic protein Bad. Ras also interacts with Raf-1 and targets it to the plasma membrane [56]. Thus, it is possible that competition between Ras and Bcl-2 for binding to limiting amounts of Raf-1 determines Raf-1's localization, substrates and effects on cell survival or death [53].

5.4. Ras

The BH4 domain of Bcl-2 is also reported to interact with activated Ras and thereby block Ras-mediated apoptotic signaling [57]. Ras is a small GTPase oncogene involved in many cell processes, including proliferation, differentiation and apoptosis, mainly through its effects on signaling pathways regulated by MAP kinase, growth factors, and Fas [58–60]. Deletion of the BH4 domain from Bcl-2 abrogates the coimmunoprecipitation of Bcl-2 with Ras and eliminates the anti-apoptotic effect of Bcl-2 in Fas-induced apoptosis.

5.5. CED-4

Both Bcl-2 and Bcl-xL interact with the non-mammalian proapoptotic protein CED-4, originally identified in *C. elegans*, and the BH4 domain appears required for this interaction [33]. CED-4 enhances CED-3-induced cell death and full length Bcl-xL, but not ΔBH4 Bcl-xL, antagonizes the apoptotic activity of CED-4. Although Apaf-1 is a CED-4 mammalian homologue, a predicted interaction between Bcl-2 and Apaf-1 has not been supported experimentally [61,62].

5.6. Paxillin

Paxillin is a focal adhesion-associated adaptor protein, serving as a docking protein to interact with focal adhesion and cytoskeleton or signal transduction proteins. It is required in embryonic development and plays critical roles in cell spreading and motility [63]. Cell adhesion determines tissue architecture during morphogenesis and inhibits apoptosis [64–66]. Recent work by Sorenson showed that the BH4 domain of Bcl-2 interacts with paxillin in lysates from embryonic kidney cells, HEK293 cells and NIH3T3 cells [67]. Amino acids 17–31 in the BH4 domain of Bcl-2 are necessary for the Bcl-2 interaction with paxillin (Fig. 1B). Tyrosines 21 and 28 in the BH4 domain are especially critical for this interaction. A BH4 domain peptide is also sufficient to interact with paxillin and disrupt nephrogenesis. Although how Bcl-2 regulates apoptosis by interacting with paxillin is still not understood, it has been proposed that Bcl-2 protects cells from apoptosis caused by loss of adhesion [67,68]. The focal adhesion kinase and paxillin complex is thought to regulate cell adhesion and migration in an integrin-mediated signaling pathway [69]. Apoptosis controls inappropriate cell positioning during three dimensional morphogenesis [64]. Bcl-2 may bypass integrin-mediated survival signals via interactions with the paxillin/focal adhesion kinase complex, circumventing the need for adhesion and thereby modulating cell adhesion and migration [68].

5.7. NF-κB

Nuclear factor κB (NF-κB), a transcription factor, plays an important antiapoptotic function in mammalian cells [70,71]. NF-κB activation is required for Bcl-2's antiapoptotic function in ventricular myocytes [72]. Also, the presence of Bcl-2-NF-κB complexes has been confirmed in nuclear fractions of NIH3T3 cells and it is thought that this interaction contributes to Bcl-2's roles in cell cycle control and apoptosis [73]. Full length Bcl-2 has been shown to enhance NF-κB's DNA binding activity, but this activity is lost when the BH4 domain is deleted from Bcl-2. Also, both the level and activity of the NF-κB inhibitor IκBα were suppressed by Bcl-2 but not by ΔBH4 Bcl-2.

5.8. IP3R

Recently we found that Bcl-2 interacts with all three subtypes of IP3R, documented by multiple experimental approaches, including coimmunoprecipitation, Blue Native Gel Electrophoresis, GST pull-down and Fluorescence Resonance Energy Transfer [13,27]. The interaction of Bcl-2 and Bcl-xL with the IP3R has been confirmed by a number of laboratories [27–31]. Although Bcl-2 is widely known to localize to mitochondria, it is also well documented on the ER where it interacts with the IP3R, an IP3 sensitive intracellular Ca^{2+} channel. The IP3R transmits Ca^{2+} from the ER lumen to the cytoplasm, elevating cytoplasmic Ca^{2+} concentration and thereby generating Ca^{2+} signals that mediate a wide range of cellular processes, including apoptosis. Through its interaction with IP3R's, Bcl-2 inhibits IP3-dependent opening of IP3R channels reconstituted in planar lipid bilayers and also inhibits IP3-dependent Ca^{2+} elevation induced by T cell receptor (TCR) activation or by a cell permeant IP3 ester.

We recently mapped the Bcl-2 interacting site to an eighty amino acid sequence within the regulatory and coupling domain of the IP3R, and based on a twenty amino acid sequence within this region developed an inhibitory peptide, referred to as peptide 2, that disrupts the Bcl-2–IP3R interaction [13] (Fig. 2). By using peptide 2 to abrogate the Bcl-2–IP3R interaction, we established that this interaction is indeed necessary for Bcl-2's inhibitory effect on IP3-mediated Ca^{2+} elevation and apoptosis in lymphocytes following TCR activation. Thus, the regulatory effect of Bcl-2 on IP3-induced Ca^{2+} elevation contributes to Bcl-2's antiapoptotic action by a mechanism different from the well-known inhibitory effect of Bcl-2 on proapoptotic members of the Bcl-2 protein family.

In recent unpublished studies, we found that the BH4 domain of Bcl-2 is necessary and sufficient to interact with the IP3R. Moreover, a peptide corresponding to the BH4 domain, when coupled with HIV-TAT to facilitate its entry into cells, inhibits IP3-dependent Ca^{2+} elevation and apoptosis in lymphocytes following TCR activation (unpublished data). These findings further indicate that Bcl-2's inhibitory effect on IP3R-mediated Ca^{2+} elevation and apoptosis is independent of other BH domains or binding with other Bcl-2 family members.

In summary, the BH4 domain of Bcl-2 interacts with a number of different factors and thereby plays an important role in apoptosis inhibition. Although our laboratory has focused mainly on the interactions of Bcl-2 and Bcl-xL with the IP3R, the other interactions discussed above may contribute together with the Bcl-2–IP3R interaction to enable Bcl-2 to regulate a wide range of signaling pathways, perhaps including pathways not directly related to apoptosis.

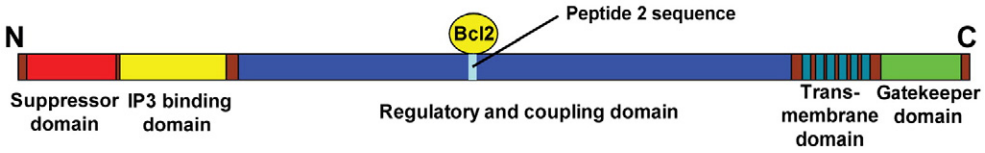

Fig. 2. Bcl-2 interacts with the regulatory and coupling domain of the IP3R. The locations of various structural and functional domains in the IP3R [96–98] are shown here, in addition to the site within the regulatory and coupling domain of the IP3R where Bcl-2 interacts. The sequence of peptide 2, which inhibits the interaction of Bcl-2 with the IP3R, was derived from the sequence of the Bcl-2 binding site on the IP3R [13].

6. Bcl-2 inhibitors

Many diseases can be attributed directly or indirectly to altered apoptosis regulation. Targeting apoptosis has become an attractive therapeutic strategy in the treatment of these disorders, especially cancer which is often associated with resistance to apoptosis, often due to elevated levels of Bcl-2 or other antiapoptotic Bcl-2 family members. A number of proteins and small molecules designed to trigger cell death have entered the clinic for use against cancer. Due to Bcl-2's antiapoptotic and oncogenic function in cancer cells, Bcl-2 draws a lot of attention as one of the major targets in apoptosis targeting therapies.

Many small molecule Bcl-2 inhibitors are in the pipeline. Genasence (Genta), an antisense oligonucleotide targeted against Bcl-2, can reduce Bcl-2's expression level [74,75]. It has entered Phase III clinical trials for chronic lymphocytic leukemia (CLL) and metastatic melanoma [76,77]. ABT-737 and ABT-263 by Abbott Pharmaceuticals [78], Obatoclax by Gemin X [79], and AT-101 by Ascenta [80] are all BH3 only protein mimetics that bind to the hydrophobic groove of Bcl-2/Bcl-xL and displace proapoptotic proteins from Bcl-2's inhibitory grip. A small molecule Bcl-2 inhibitor by Infinity Pharmaceuticals also interrupts the interactions between Bcl-2 and its proapoptotic binding partners. Some other natural and chemical inhibitors of Bcl-2, including HA14-1 [81], BH3I-I [82], chelerythrine [83], and gossypol [84] appear mainly to regulate the Bcl-2 interaction with other Bcl-2 family members. As described before, the site targeted by the novel therapeutics is the hydrophobic groove formed by the BH1, BH2 and BH3 domains. Surprisingly, none of them target the BH4 domain of Bcl-2 to antagonize Bcl-2's antiapoptotic function, even though the BH4 domain contributes to the antiapoptotic function of Bcl-2 as summarized as above.

The concept that the Bcl-2 BH4 domain may be a worthy target arises in part from our studies of the Bcl-2–IP3R interaction, which is mediated through the BH4 domain. As discussed earlier, a 20 amino acid peptide (peptide 2), mimicking the Bcl-2 binding site sequence on the IP3R, abrogates the Bcl-2–IP3R interaction, thereby reversing Bcl-2's inhibitory effect on IP3-mediated Ca^{2+} signaling and apoptosis. Peptide 2 is derived from the sequence of IP3R that binds to the BH4 domain of Bcl-2. Peptide 2 does not interfere with the interaction of Bcl-2 with Bim [13]. Thus, peptide 2 targets the BH4 domain of Bcl-2 instead of the hydrophobic groove formed by BH1, 2 and 3 domains. Peptide 2's proapoptotic effect makes it of potential value in cancer therapy.

As an initial test of this hypothesis, we investigated the effect of peptide 2 in chronic lymphocytic leukemia (CLL), a common human malignancy associated with elevated levels of Bcl-2. CLL cells are "addicted to Bcl-2" because they have elevated levels of both BH3-only proteins and Bcl-2 [8]. Consequently, CLL cells undergo apoptosis if the ability of Bcl-2 to bind and inhibit BH3-only proteins is abrogated by treatment with the BH3-mimetic ABT737 [8]. Although peptide 2 by itself was not toxic to primary CLL cells, peptide 2 significantly enhanced ABT-737 induced apoptosis (Fig. 3). It would appear that a combinatory inhibitory effect is achieved by simultaneously targeting two different sites on Bcl-2. Although this preliminary study involved CLL cells from only six patients, the results are promising enough to cast light on the potential importance of the BH4 domain as a therapeutic target. Also, it is not possible at this point to be sure whether the mechanism of action of peptide 2 in this context is the disruption of Bcl-2's interaction with the IP3R or one of the other BH4 domain interacting partners described above.

7. BH4 peptide has antiapoptotic function

Interestingly, although the BH4 domain has not previously been targeted to reverse Bcl-2's antiapoptotic action, the BH4 domain peptide is used to mimic Bcl-2 as an antiapoptotic reagent in many

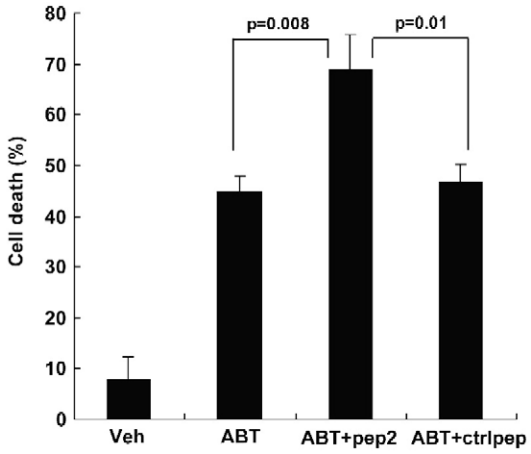

Fig. 3. Peptide 2 enhances ABT-737-induced apoptosis in CLL cells. Lymphocytes were freshly separated from heparinized peripheral blood obtained from adult patients with chronic lymphocytic leukemia meeting standard diagnostic guidelines. We conformed to all guidelines and regulations in accordance with Internal Review Board protocols ICC2902/11-02-28 (Case Western Reserve University Cancer Center/University Hospitals of Cleveland Ireland Cancer Center). Cells were separated by centrifugation through Ficoll–Hypaque and cultured in RPMI medium (10% fetal bovine serum) at a density of 2.0×10^6 cells/ml. CLL cells were treated in vitro with 2 μM ABT-737 in the presence or absence of 5 μM TAT-peptide 2 (pep2) or TAT-control peptide (ctrlpep). Cell death was detected by trypan blue staining after 24 h treatment. Six individual experiments were analyzed by the two-tailed Student's *t* test, using a significance level of $p<0.05$.

apoptosis models. Shimizu et al used a TAT-BH4 peptide to facilitate BH4 peptide entry into cells and found that the TAT-BH4 peptide prevents Ca^{2+}-induced loss of mitochondrial membrane potential and cytochrome *c* release [49]. Moreover, the TAT-BH4 peptide inhibited X-ray- and VP-16 (Etoposide)-induced apoptosis, but not tunicamycin-induced apoptosis in PC-12 or Hela cells. Injection of TAT-BH4 peptide into the peritoneum of C57BL6J mice conferred protection against X-ray irradiation-induced cell death in the small intestine [85]. Also, TAT-BH4 peptide suppressed anti-Fas induced fulminant hepatitis [85] and improved ischemia–reperfusion-induced cardiac dysfunction, therefore attenuating ischemia–reperfusion injury in the rat heart [86]. Other groups found that BH4 peptide inhibits oxidative stress induced coronary endothelial cell apoptosis [87], human islet cell apoptosis, staurosporine and serum deprivation-induced apoptosis [88,89], radiation-induced apoptosis in human T lymphocytes and B cells [90], beta-amyloid peptide-induced apoptosis, and sepsis-induced apoptosis [91]. In addition, BH4 peptide's convenient delivery into cells and antiapoptotic effect endow it protective roles in vivo in spinal cord injury [92], islet transplantation [88], neurotoxicity and hippocampal damage [93].

Recently, IP3R mediated Ca^{2+} release has been implicated as contributing to the pathogenesis of Alzheimer's disease [94]. Interestingly, TAT-BH4 peptide reduces the toxic effect of beta-amyloid peptide on capillary endothelium [95]. The mechanism of the protective action of TAT-BH4 in Alzheimer's disease is not fully elucidated. We have found TAT-BH4 peptide, like full length Bcl-2, inhibits IP3R-mediated Ca^{2+} release from the ER and apoptosis in T cells (unpublished data). This raises the possibility that a similar mechanism might be relevant in Alzheimer's disease.

In summary, there is considerable interest in development of antiapoptotic therapies for diseases associated with accelerated cell death, based on the BH4 domain of Bcl-2 or Bcl-xL, even though the role of the BH4 domain in Bcl-2/Bcl-xL function has not been fully elucidated.

8. BH4 domain structure

In view of BH4 domain's importance as a potential target to regulate Ca^{2+} signaling and apoptosis, the concept of blocking BH4 domain interactions with other proteins involved in regulating or

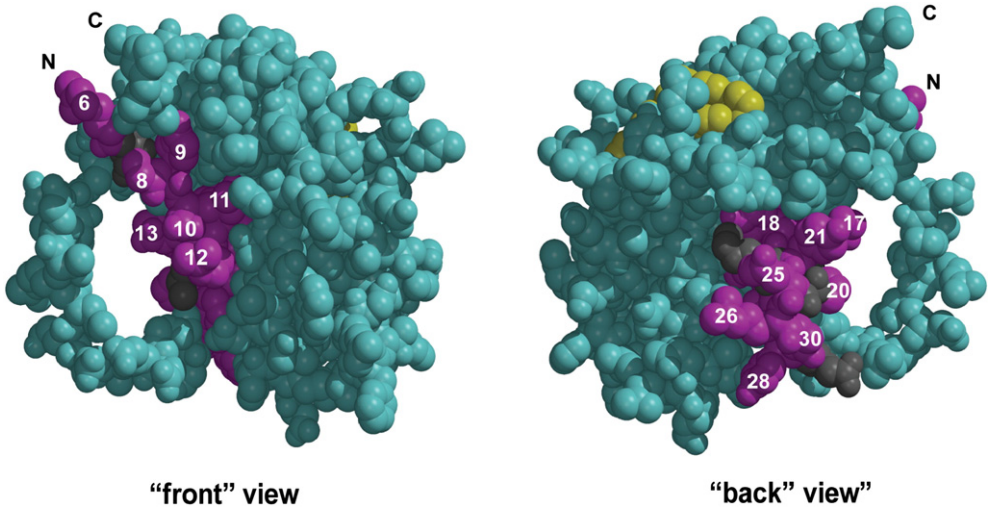

Fig. 4. BH4 domain residues in the Bcl-2 structure. The NMR structure of Bcl-2 bound to a sulfonamide inhibitor is shown as space-filling spheres. BH4 domain residues which are most conserved in the 7 closest homolog sequences are in purple (conserved residues 6, 8–15, 17–21, 23, 25–28, and 30) while those which are not conserved are gray. The remainder of the Bcl-2 structure is cyan, with the bound sulfonamide inhibitor in yellow. (Figure drawn using MolScript [99] and Raster3D [100] using coordinates with PDB accession code 1ysw [78]).

mediating apoptosis (e.g., IP3R, calcineurin, paxillin) by small molecules or peptides, as illustrated by peptide 2, provides another opportunity to design Bcl-2 inhibitors. Not only does the BH4 domain's role in apoptosis regulation provide an opportunity to develop therapeutics designed to enhance apoptosis by abrogating Bcl-2's antiapoptotic function, but the ability of the BH4 peptide by itself to inhibit apoptosis encourages efforts to identify or engineer molecules that mimic the BH4 domain and thus have antiapoptotic function similar to that of full length Bcl-2/Bcl-xL. These would likely prove to be useful in diseases associated with accelerated cell death. Based on what is known about the structure and location of the BH4 domain in Bcl-2 these goals should be feasible.

The BH4 domain is an α-helical region located at the N-terminus of Bcl-2. Based on conservation and surface accessibility, the Bcl-2 BH4 domain residues predicted to be most like involved in binding other proteins are D10 and R12 (on one face) and H20, Y21, Q25, R26, and Y28 (on the opposite face) (Fig. 4). These residues are conserved in BH4 domains of seven of the closest mammalian Bcl-2 family member sequences and have highly accessible exposed side chains available for specific binding to other molecules. A positive and a negative residue are located on one face, and positively charged residues on the opposite face which may contribute to ionic or hydrogen bond interactions. The residues R12, I14, V15, Y18, I19 and L23 have been reported indispensable for the anti-apoptotic activity of Bcl-2 [33,36].

Notably, the hydrophobic groove formed by the BH1-3 domains, responsible for interaction with BH3-only proteins and targeted therapeutically by BH3-mimetics, is separate from the BH4 domain in the three dimensional structure of Bcl-2. Thus, it will be interesting to explore targeting the predicted binding sites of the BH4 domain to develop therapeutics that act by a different mechanism to inhibit Bcl-2's antiapoptotic activity.

9. Summary

The BH4 domain is present only in antiapoptotic members of the Bcl-2 protein family and thus is a major distinguishing feature that separates the antiapoptotic and proapoptotic family members at a molecular level. Although this is the case, exploring the BH4 domain for its therapeutic potential has lagged far behind the concentrated efforts focused on the interaction of antiapoptotic family members with proapoptotic family members mediated through the BH1, BH2 and BH3 domains. The BH4 domain is not only a promising target for small molecule therapeutics intended to reverse the antiapoptotic functions of Bcl-2 and Bcl-xL, but also a useful model for therapeutics intended to inhibit apoptosis based on considerable evidence that a BH4 peptide itself inhibits proapoptotic Ca^{2+} signals and apoptosis in a variety of settings.

The BH4 domain-mediated interactions with IP3R and other apoptotic regulators is another dimension of Bcl-2's function that is not sufficiently understood. The development of peptide inhibitors or small molecule peptide mimetics directed at the BH4 domain of Bcl-2 will be one way to further elucidate the function of the BH4 domain. This is illustrated by our recent development of peptide 2 based on detailed analysis of the interaction of Bcl-2 with the IP3R. The use of peptide 2 has already established the importance of the Bcl-2–IP3R interaction in regulating Ca^{2+} signals and apoptosis, and even suggested potential therapeutic utility of targeting the BH4 domain for treatment of diseases associated with elevated Bcl-2. Perhaps in the future BH4 domain-based Bcl-2 targeting strategies combined with BH3 mimetic strategies will inhibit Bcl-2's function more efficiently than either alone in cancer therapy. Alternatively, perhaps therapeutics based on the intrinsic antiapoptotic activity of the BH4 domain will be useful for treatment of diseases associated with accelerated cell death.

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